Localization

miRCURY LNA™ microRNA ISH Optimization Kit (FFPE)

Instruction manual v2.0

for product # 90000, 90001, 90002, 90003, 90004, 90005, 90007, 90008, 90009

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Literature citations

Please refer to miRCURY LNA™ microRNA Detection probes and ISH optimization kit when describing a procedure for publication using this product or to the following article: Robust One-day *in situ* hybridization protocol for detection of microRNA in paraffin samples using LNA probes. Jørgensen S, Baker A, Møller S, Nielsen BS. Methods (2010), 52,373-381.

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Abbreviations. FFPE: formalin-fixed and paraffin-embedded. ISH: *In situ* hybridization. LNA™: locked nucleic acid. Prot-K: Proteinase-K. RT: room temperature. AP: alkaline phosphatase. DIG: digoxigenin. PFA: paraformaldehyde.

Product summary

Product summary

The miRCURY LNA™ microRNA ISH Optimization Kit (FFPE) provides the user with reagents and recommendations to ensure the best starting point for successful microRNA *in situ* hybridizations (ISH) on formalin-fixed paraffin embedded (FFPE) tissue samples. The kit contains 3 digoxigenin (DIG)-labeled probes: one double- (5' and 3')-DIG labeled probe for a known cell-specific microRNA, one double-(5' and 3')-DIG labeled scrambled probe to use as negative control, and one 5'-DIG labeled probe against U6 snRNA for use in the early-phase assay set-up. In addition, the kit contains a formamide-free hybridization buffer developed specifically for miRCURY LNA™ Detection probe-based ISH. The included Proteinase-K will allow the user to optimize the Proteinase-K treatment for optimal retention of the microRNA target.

The accompanying One-day microRNA ISH protocol minimizes time-consuming optimization steps and enables a fast and optimal microRNA ISH analysis using a colorimetric antibody-based development system for the DIG labeled probes. In addition, the Instruction Manual carefully covers each step of the FFPE ISH procedure, including tissue sectioning, incubation intervals and temperatures, miRCURY LNA™ microRNA Detection probe concentrations and substrate incubation. The manual further contains a list of recommended equipment and reagents required to establish and optimize microRNA ISH in the lab.

Kit content

Reagent	Vol.	Conc.	RNA T_m
LNA™ microRNA probe, double-DIG labeled	40µL	25µM	See datasheet
LNA™ scrambled microRNA probe, double-DIG labeled (5'-gtgtaacacgtctatacgccca-3')	40µL	25µM	87°C
LNA™ U6 snRNA probe, 5' DIG-labeled (5'-cacgaatttgcgtgtcatcctt-3')	40µL	0.5µM	84°C
microRNA ISH buffer (2x)	25mL		
Proteinase-K, lyophilized	12mg		

Shipping and storage

Upon receipt

The kit is shipped at room temperature. Immediately upon receipt, remove the miRCURY LNA™ microRNA ISH buffer from the box and store at 4°C. Store the DIG-labeled miRCURY LNA™ microRNA Detection probes and Proteinase-K in the box at -20°C or below. Under these conditions the probes are stable for at least 6 months. It is recommended to store the probes in aliquots and to avoid multiple freeze-thaw cycles (see recommendations below). Do not store in frost-free freezer with automatic thaw-freeze.

Before First Use

- Proteinase-K stock: reconstitute to 20 mg/mL by adding 600 µL 10mM Tris-HCl, pH7.5 (RNase-free). Store appropriate aliquots at -20°C.
- DIG-labeled LNA™ probes: The following options are possible:
  1) The probes may be stored at 4°C if used within 4 weeks.
  2) Prepare aliquots to be stored at -20°C or below and avoid multiple freeze-thaw cycles. Example: divide the LNA™ Detection probes into 5µL or 10µL aliquots into non-stick RNase-free tubes.
  3) Prepare pre-diluted probe aliquots to be stored at -20°C or below in the the microRNA ISH buffer. This option is only recommended once the optimal probe concentration has been determined. For details of how to make 1x ISH buffer

and how to denature and dilute the probes, please see page 15. On the day of use, thaw the pre-diluted probe to room temperature and apply directly to the sections. Note: Probe concentration must be optimized (see Tip 3, page 24).

Additional required material

ISH protocols vary extensively due to different equipment set-up and laboratory routines. This one-day miRCURY LNA™ microRNA ISH protocol details the process of manual ISH on formalin-fixed and paraffin embedded tissue samples using double-DIG labeled miRCURY LNA™ microRNA Detection probes.

For the ISH steps we recommend a hybridization station that allows precise and rapid temperature adjustments, e.g. Dako Hybridizer. This protocol is developed using a hybridization station, but if unavailable, conventional hybridization ovens may be used (see details in FAQs on page 29).

For the immunohistochemical steps Exiqon has had good experience with both horizontal humidifying chambers and Shandon's Sequenza Slide Rack systems.

The chromogenic ISH assay is based on the use of DIG-labeled probes and therefore requires proper detection reagents (e.g. alkaline phosphatase-conjugated anti-DIG and NBT-BCIP substrate).

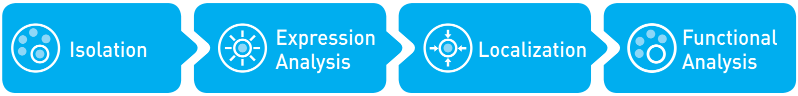
Required reagents and equipment, not supplied

In addition to the reagents supplied in the miRCURY LNA™ microRNA ISH Optimization Kit, a series of reagents as well equipment is needed to perform the ISH experiments as described in the protocol. The list below includes recommendations for specific products that have been shown to work well with the microRNA ISH protocol (details of how to prepare buffers and reagents can be found on page 13-16):

- Double-DIG-labeled miRCURY LNA™ Detection probes for your microRNA(s) of interest (Exiqon)
- Sheep anti-DIG-AP (Roche, Cat. No. 11 093 274 910)
- Sheep serum (Jackson Immunoresearch, Cat. No. 013-000-121)
- NBT/BCIP ready-to-use tablets (Roche, Cat. No. 11 697 471 001) or equivalent
- Levamisole (Fluka, Cat. No. 31742 or equivalent)
- Rubber cement, Fixogum (MP Biomedicals, Cat No 11FIXO0050) or equivalent
- Nuclear counter stain, Nuclear Fast Red™ (Vector laboratories, Cat. No. H-3403) or equivalent
- Mounting medium, Eukitt® (VWR, Cat. No. 361894G)
- RNaseZap® (Ambion)
- Hybridizer, e.g. Dako Hybridizer, Vysis' ThermoBrite, or Invitrogen's Spotlight Hybridizer
- Humidifying chamber or equivalent for immunohistochemical detection
- Superfrost®Plus slides
- Cover slides
- Slide rack(s) and several glass jars for deparaffinization, dehydration, and washes
- Xylene (for de-paraffination)
- Ethanol (for hydration and dehydration)
- PBS, sterile
- SSC buffer, ultrapure
- 1M Tris-HCl, pH 7.4
- 0.5M EDTA
- 5M NaCl
- Tween-20 (Sigma, cat no. P1379)
- 30% BSA (Sigma, cat no. A9576)
- For KTBT buffer: Tris-HCl, NaCl and KCl (see page 14)
- Dako Pen (or equivalent hydrophobic PAP pen)
- Non-stick RNase free microfuge tubes
- Sterile filter tips

Related products

Exiqon offers a broad variety of products enabling new discoveries concerning the isolation, expression, function and spatial distribution of microRNAs:



miRCURY LNA™ microRNA ISH Buffer Set

Hybridation buffer and Proteinase K for continuation of the optimized ISH procedure with miRCURY LNA™ microRNA Detecion probes.

miRCURY™ RNA Isolation Kits

Get high quality total RNA suitable for miRCURY LNA™ microRNA Array analysis in as little as 20 minutes. Protocols are available for a large number of sample types and organisms.

miRCURY LNA™ microRNA Array, microarray kit

Pre-printed miRCURY LNA™ microRNA Array microarray slides, for hsa, mmu & rno. Kit includes hybridization and wash buffers as well as synthetic spike-in microRNAs.

miRCURY LNA™ microRNA Power and Hi-Power labeling kit

For fluorescent labeling of microRNAs from total RNA samples ready for array hybridization.

miRCURY LNA™ microRNA Array, ready-to-spot probe set

Ready-to-spot oligos for direct printing of arrays, or coupling in bead-based applications.

miRCURY LNA™ Universal RT microRNA PCR

Exiqon's microRNA qPCR system offers the best available combination of performance and ease-of-use on the microRNA real-time PCR market. The combination of a Universal RT reaction and LNA™-enhanced PCR primers results in unmatched sensitivity and specifi city. The Ready-to-use microRNA PCR panels enable fast and easy microRNA expression profiling. Pre-validated individual assays and custom assays are also available.

miRCURY LNA™ microRNA Detection Probes

For *in situ* hybridization and northern blotting of all microRNAs.

miRCURY LNA™ microRNA Inhibitors and Power Inhibitors

Unravel the function of microRNAs by microRNA inhibition. Sophisticated LNA™ design ensures potent inhibition of all microRNAs regardless of their GC content. Chemically modified, highly stable Power Inhibitors for unrivalled potency.

miRCURY LNA™ microRNA Inhibitor Library

For genome-wide high throughput screening of microRNA function.

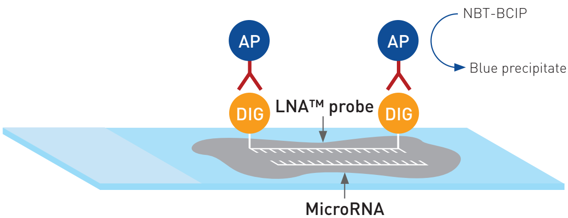
Product description

In situ hybridization (ISH) is a powerful technique and the most common method for visualizing gene expression and localization in specific tissue and cell types. The technology is far from trivial and is often a very time-consuming and difficult procedure requiring many steps of protocol optimizing to achieve satisfactory ISH results. Detection of microRNA by conventional ISH analysis is no exception.

The miRCURY LNA™ microRNA ISH Optimization kit (FFPE) offers a fast and robust procedure for an easy implementation of microRNA ISH analysis requiring a minimum of optimization. The microRNA ISH buffer is specifically developed for use with the double-DIG labeled miRCURY LNA™ microRNA Detection probes. Used in combination, this provides the best available method for specific and sensitive detection of microRNA expression by ISH in FFPE sections of any tissue specimen.

The ISH protocol is designed for detection of microRNA in FFPE tissue sections and takes advantage of the use of the non-mammalian hapten digoxigenin (DIG), and has been optimized to fit into a one-day experimental set-up. During the protocol the microRNAs are demasked using Proteinase-K, which allows the access of double-DIG-labeled LNA™ probes to hybridize to the microRNA sequence (Figure 1). The digoxigenins can then be recognized by a specific anti-DIG antibody that is directly conjugated with the enzyme Alkaline Phosphatase (AP). AP converts the soluble substrates 4-nitro-blue tetrazolium (NBT) and 5-bromo-4-chloro-3'-indolylphosphate (BCIP) into a water and alcohol insoluble dark-blue NBT-BCIP precipitate. Finally, the nuclear counter stain is applied to the sections to allow better histological resolution.

Figure 1

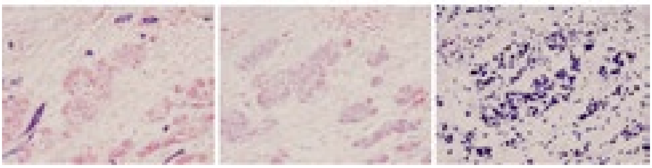


Optimization of the ISH procedure is divided into three steps:

1. Optimization of the protocol parameters with the LNA™ U6 snRNA probe by adjustment of hybridization temperature and Proteinase K treatment.
2. Control study using the optimized protocol parameters with the kit-specific double-DIG LNA™ microRNA probe and scrambled negative control probe (a strong specific ISH signal should be obtained).
3. Detection of the microRNA of interest using the appropriate miRCURY LNA™ microRNA Detection probe.

Figure 2 shows a typical result of the microRNA ISH procedure. In this case, specific miR-126 ISH signal is seen in endothelial cells, as expected no signal is observed with LNA™ scrambled microRNA probe, and overall nuclear staining is seen with LNA™ U6 snRNA probe.

Figure 2



miR-126, 40nM scrambled, 40nM U6 snRNA, 0.1nM

Figure 2. *In situ* hybridization on consecutive sections from FFPE tissue sample with human breast cancer using the miRCURY LNA™ microRNA ISH Optimization kit 5 (FFPE) with the positive control miRCURY LNA™ Detection probe, miR-126.

Once optimized, the protocol allows exceptionally clear and specific detection of microRNA at the cellular and partly subcellular level due to high signal-to-noise ratio. An excellent histological resolution is obtained in a wide range of tissue samples. In fact, eight different kits are available, each with a unique positive control miRCURY LNA™ microRNA Detection probe targeting a specific microRNA known to be highly expressed in a distinct organ or cell type. This makes the kit ideal for a variety of applications ranging from study of microRNA localization, developmental microRNA regulation, examination of functional studies over diagnostic and prognostic biomarker discovery in clinical specimens and other biopsy material. The robustness of the procedure makes it advantageous for both high-throughput ISH analysis as well as individual microRNA localization studies.

Protocol

Before starting the experiment

In order to ensure that the lab, equipment and reagents for the microRNA ISH procedure are in place before starting the experiment, it is recommended to go through the following sequence of steps:

1) Prepare and store kit reagents (page 5).
2) Establish histology Lab environment for RNA work (page 12-13).
3) Prepare samples (including fixation of tissue and FFPE preparations, page 13).
4) Cut FFPE sections using Tissue Sectioning Guidelines (page 23).
5) Prepare reagents and buffers (page 14-16).
6) Become confident with the steps in the One-day miRCURY LNA™ microRNA ISH protocol (page 17-22).
7) Evaluate appropriate Proteinase-K treatment range for each sample type.
8) Determine ISH sensitivity level using the LNA™, U6 snRNA probe. It may be necessary to repeat steps 7 and 8 to gradually improve the performance of the protocol.
9) Run the microRNA ISH protocol with the LNA™ microRNA probe and LNA™ scrambled microRNA probe.
10) Optimize hybridization temperature and probe concentration for each probe.

An overview of the workflow for the One-day miRCURY LNA™ microRNA ISH protocol is shown on page 17.

Important – cautions for RNA work

RNA work requires specific handling and precautions to prevent RNase contamination of the reagents and degradation of the RNA sample.

Every step in the microRNA ISH procedure including tissue sectioning and DIG-detection, must take place in a clean and nuclease free environment. We recommend that all surfaces are cleaned with RNaseZap®, RNase Away or other RNase removal solution. Wear gloves during the entire process, and only use RNase decontaminated glassware. All buffers and reagents should be prepared using RNase-free water only, e.g. RNase-grade Milli-Q water or DEPC-treated water, and be autoclaved if specified.

FFPE sample requirements and comments on tissue fixation

MicroRNAs are like other RNA fragile molecules sensitive to degradation. Fast and sufficient fixation of tissue specimens is therefore important for successful ISH analyses. For ISH analysis in human specimens, standard overnight fixation in neutral-buffered formalin followed by paraffin embedding often works well. For studies in mouse tissues, perfusion fixation with 4% fresh PFA is recommended. Consult animal care guidelines before setting up this protocol, see e.g. The Laboratory Mouse by Mark A. Suckow, Peggy Danneman, Cory Brayton (CRC Press) or Pathology of Genetically Engineered Mice by Jerrold Michael Ward, Joel F. Mahler, Robert R. Maronpot (Iowa State University Press). Optimization of the assay performance should preferably be based on analysis of at least 4 FFPE blocks.

For detailed guidelines to Tissue Sectioning, please see Tip 1, page 23.

See tip 1

Glassware

In order to reduce potential RNase contamination of glassware, it is recommended to autoclave all glassware or to heat-treat all glassware for 8 hours at 180°C. Prior to the heat-treatment, it is recommended to wrap all items in aluminum foil, including appropriate stacks of cover slips. Keep apart from untreated glassware.

Preparation of reagents and buffers

In addition to the hybridization buffer and Proteinase-K buffer supplied with the microRNA ISH Optimization Kit a number of other reagents and buffers need to be prepared prior to initiating the microRNA ISH experiment (see page 7 for a list of recommended materials). Recipes for preparation of required reagents and buffers are listed in Table 1-3.

Please note that many reagents should be freshly prepared on the day of the experiment or even immediately before use.

Table 1. Reagents needed during the ISH procedure

Antibody blocking solution	PBS, 0.1% Tween, 2% Sheep serum, 1% BSA, (see table 3 for details)
Antibody dilutant solution	PBS, 0.05% Tween, 1% sheep serum, 1% BSA (see table 3 for details)
Sheep-anti-DIG-AP	See table 3
NBT/BCIP ready-to-use tablets	See table 3
Levamisole	For blocking endogenous AP activity. Prepare a 100 mM stock
Nuclear Fast Red™	Nuclear counter stain

Table 2. Buffers and stocks to prepare and autoclave* prior to the microRNA ISH experiment

Proteinase-K buffer	To 900 mL RNase-free water add 5 mL of 1 M Tris-HCl (pH7.4) 2 mL of 0.5 M EDTA 0.2 mL of 5 M NaCl Adjust volume to 1000 mL. Autoclave*.
20xSSC pH 7.0	If purchased as RNase-free then leave as is.
SSC solutions	5xSSC (1 L=250 mL 20xSSC + 750 mL water) 1xSSC (1 L=50 mL 20xSSC + 950 mL water) 0.2xSSC (1 L=10 mL 20xSSC + 990 mL water) Autoclave
PBS-T (0,1%), pH7.4	Add 1 mL of Tween-20 to 1 L of PBS. Autoclave*.
KTBT (AP stop solution)	To 900 mL RNase-free water add 7.9g Tris-HCl (50mM) 8.7g NaCl (150mM) 0.75g KCl (10mM) Adjust volume to 1000mL. Do not adjust pH. Autoclave*.

*autoclave buffers where listed to minimize RNase activity. RNaseAlert® Lab test Kit (Ambion) is an easy and fast test that is recommended for optional testing of potential RNase activity in buffers and reagents.

Table 3. Reagents to prepare on the day of the experiment

Proteinase-K reagent	Prepare immediately before use. For a Proteinase-K concentration of 15 µg/mL: Add 7.5 µL Proteinase K stock to 10 mL Proteinase K buffer (prepared in Table 2). See further recommendations in Tip 2, page 23.
Hybridization mix (microRNA ISH buffer and LNA™ Detection probes)	1) Dilute the 2x microRNA ISH buffer 1:1 with RNase-free water, e.g. mix 1 mL 2x microRNA ISH buffer with 1 mL RNAse-free water to give 2 mL 1x buffer. 2) For each probe to be used in the experiment, place the appropriate amount of LNA™ probe in a 2 mL non-stick RNase-free tube (see table below). 3) Denature the probes at 90°C for 4 minutes. 4) Place the tubes in table-top microfuge and spin down shortly. 5) Immediately add the 2 mL 1x microRNA ISH buffer to each of the tubes with the different LNA™ probes.

Probe	Final probe conc.	Probe vol.	Dilution factor	1x ISH buffer vol.
LNA™ U6 snRNA (0,5µM)	1 nM	4 µl	1:500	2 ml
LNA™ microRNA probe (25µM)*	20 nM	1,6 µl	1:1250	2 ml
LNA™ microRNA probe (25µM)*	40 nM	3,2 µl	1:625	2 ml
LNA™ scrambled microRNA probe (25µM)	40 nM	3,2 µl	1:625	2 ml

* Suggested starting concentrations for microRNA probes can be found in table 9, page 25.
Note: left over hybridization mix can be stored at -20°C and will be stable for up to 6 months, avoid multiple freeze-thaw cycles.

Continued next page.

Antibody blocking and dilutant solutions	1) To make 10mL blocking and 10mL dilutant solution start with 15mL PBS-T (see table 2) and add 300µL Sheep serum (2% final concentration). Label the tube "Blocking solution" 2) Remove 5 mL from the tube in step 1, place in a new tube and label "Dilutant solution". 3) To the tube labeled Blocking solution, add 330µl 30% BSA to give a final concentration of 1%. The blocking solution is now ready to use. 4) To the tube labeled Dilutant solution, add 5 mL PBS (to give 0.05%Tween and 1% sheep serum final concentration) and 330µl 30% BSA to give 1% final concentration . The diluant solution is now ready for use.
Anti-DIG reagent	Dilute the sheep-anti-DIG-AP antibody 1:800 in Antibody dilutant solution (see above). (range 1:500-1:2000).
AP substrate	Immediately prior to use, dissolve a NBT-BCIP tablet in Milli-Q water according to the manufacturer's instructions. Add Levamisol to a final concentration of 0.2 mM. Example: if 10 mL AP substrate is prepared, then add 20 µL Levamisol stock. Protect from light before and during incubation.

Workflow overview - One-day microRNA ISH protocol

Below is an overview of the workflow for the microRNA ISH protocol for FFPE samples. The numbers refer to each of the steps in the protocol, see details in the protocol section, page 18-22. The workflow can be followed for both the initial protocol optimization with the LNA™ probes provided with the kit and the subsequent specific microRNA detection.

Process*	Step	Equipment	Time	Temperature
Deparaffination	1	Slide rack and Jars	40 min.	Room Temperature
Proteinase-K	2	Hybridizer	10 min.	37°C
Dehydration	4	Slide rack and Jars	20 min.	Room Temperature
In Situ hybridization	5	Hybridizer	60 min.	50-60°C
Stringent washes	7	Water bath	30 min.	50-60°C
Blocking	9	IHC staining racks	15 min.	Room Temperature
Anti-DIG/AP	10		60 min.	
AP reaction	12	IHC staining racks in Oven	120 min.	30°C
Counter stain	15	IHC staining racks	10 min.	Room Temperature
Dehydration	17	Slide rack and Jars	10 min.	
Mounting	18		5 min.	

* PBS washing steps are excluded from the overview. Total time required is 7 hours.

One-day microRNA ISH protocol

The protocol describes every step in the microRNA ISH analysis. When setting up the microRNA ISH experiment for the first time it is recommended to follow the three optimization steps:

1) First, optimize the protocol parameters with the LNA™ U6 snRNA probe by adjustment of hybridization temperature and Proteinase K treatment.
2) Conduct control study using the optimized protocol parameters with the double-DIG LNA™ microRNA positive and negative control probes. Adjust hybridization temperature and Proteinase K treatment to obtain a strong specific microRNA ISH signal.
3) Finally, detect the microRNA(s) of interest using the appropriate miRCURY LNA™ microRNA Detection probe(s) with the defined protocol parameters keeping in mind that the hybridization temperature may need to be adjusted.

Step 1
Deparaffinize slides in xylene and ethanol

Deparaffinize slides in xylene and ethanol solutions at room temperature (RT) by placing slides with sections in a slide rack, and then move from glass jar to glass jar according to Table 4 ending up in PBS.

Table 4

Step	Solvent	Duration
1	Xylene	5 min.
2	Xylene	5 min.
3	Xylene	5 min.
4	99.9% Ethanol	Immerse 10 times
5	99.9% Ethanol	Immerse 10 times
6	99.9% Ethanol	5 min.
7	96% Ethanol	Immerse 10 times
8	96% Ethanol	5 min.
9	70% Ethanol	Immerse 10 times
10	70% Ethanol	5 min.
11	PBS	2-5 min.

Step 2
Incubate with Proteinase-K for 10 min. at 37°C

Immediately before use, add Proteinase-K to Proteinase-K buffer (see Table 2 & 3). Place slides on a flat surface and apply approximately 300 µL/slide to fully cover the section and incubate slides for 10 min. at 37°C for example in a Dako Hybridizer. If the Hybridizer is employed then remove the humidifying strip inserts. The Proteinase-K concentration range must be optimized for individual tissues (see Tip 2).

See tip 2

Step 3
Place slides in PBS

Place slides into a slide rack inside a jar with PBS, wash twice in PBS.

Step 4
Dehydrate slides

Dehydrate slides in new ethanol solutions according to Table 5. Air-dry the slides on clean paper towels app. 15 min.

Table 5

Step	Solvent	Duration
1	70% Ethanol	Immerse 10 times
2	70% Ethanol	1 min.
3	96% Ethanol	Immerse 10 times
4	96% Ethanol	1 min.
5	99.9% Ethanol	Immerse 10 times
6	99.9% Ethanol	1 min.

Step 5
Apply hybridization mix and hybridize for 1 hour at 50-60°C

Place slides on a flat surface and apply 25 µL hybridization mix as prepared in Table 3. For initial protocol optimization, probe concentrations could be:
a) 1 nM LNA™ U6 snRNA probe
b) 40 nM double-DIG LNA™ microRNA probe
The probe concentration will need to be optimized for optimal microRNA ISH signal. See Tip 3.
Avoid touching the tissue sections with the pipette tip. Then apply a sterile cover glass onto each section, carefully avoiding air bubbles, and seal along all four edges with Fixogum (rubber cement). Place the slides in the Hybridizer and start a program hybridizing for 1 hour. Hybridization temperature must be optimized for individual probes, see Tip 4).

See tip 3

See tip 4

Step 6
Disassemble slide and coverglass

Remove Fixogum using tweezers. Avoid sliding the coverslip, which may damage the tissue. Then, carefully detach cover glass and place the slides into a slide rack placed within a glass jar containing 5xSSC at RT.

Step 7
Wash slides in SSC buffers

Wash slides in glass jars according to Table 6. To ensure sufficient stringency perform the washes in glass jars placed in a water bath set to the hybridization temperature.

Table 6

Step	Buffer	Duration	Temperature
1	5xSSC	5 min.	Hyb temp
2	1xSSC	5 min.	Hyb temp
3	1xSSC	5 min.	Hyb temp
4	0.2xSSC	5 min.	Hyb temp
5	0.2xSSC	5 min.	Hyb temp
6	0.2xSSC	5 min.	RT

Step 8 Apply hydrophobic barrier	Transfer slides to glass jars with PBS. Apply a hydrophobic barrier around tissue sections using a DAKO-Pen following the manufacturer's instructions. Tissue sections are not allowed to dry out during this and the subsequent immunohistochemistry steps. Alternatively, if Shandon Slide Racks are employed, then assemble slides on coverplates using PBS-T.
Step 9 Incubate with blocking solution for 15 min.	Place the slides in a humidifying chamber and incubate with blocking solution for 15 min. at RT. **Important:** Steps 9-16 are carried out in the humidifying chamber or in Shandon Slide Racks.
Step 10 Apply anti-DIG reagent for 60 min.	Remove blocking solution and apply anti-DIG reagent (sheep anti-DIG-AP at 1:800 in antibody dilutant, see table 3) and incubate for 60 minutes at RT.
Step 11 3x3 min. wash in PBS-T	Wash the slides 3x3 minutes with PBS-T.
Step 12 Incubate with AP substrate for 2 hours at 30°C	Apply freshly prepared AP substrate to the sections (see Table 3) and incubate slides for 2 hours at 30°C in the humidifying chamber. Protect from light during development.
Step 13 Incubate slides in KTBT buffer 2x5 minutes	Incubate slides in KTBT buffer 2x5 minutes to stop the reaction.

Step 14
Wash with water, 2x1 min.

Wash with water, 2x1 minutes.

Step 15
Counter stain with Nuclear Fast Red™

Apply 200 µL Nuclear Fast Red™ (nuclear counter stain) for 1 minute for nuclear counter staining.

Step 16
Rinse in tap water for 10 min.

Remove slides from the humidifying chamber to a slide rack placed within a glass jar containing tap water. Carefully rinse the slides with running tap water for app. 10 min.

Step 17
Dehydrate slides

Dehydrate slides in ethanol solutions according to Table 7. Place the slides on clean paper towels.

Table 7

Step	Solvent	Duration
1	70% Ethanol	Immerse 10 times
2	70% Ethanol	1 min.
3	96% Ethanol	Immerse 10 times
4	96% Ethanol	1 min.
5	99.9% Ethanol	Immerse 10 times
6	99.9% Ethanol	1 min.

Step 18
Mount slides

Mount the slides directly with 1-2 drops of mounting medium (Eukitt®). Avoid air-drying sections at this step.

Step 19
Microscopy

Allow precipitate to settle overnight and analyze results by light microscopy the subsequent day.

Tips and troubleshooting

Tip 1

Tissue Sectioning Guidelines

It is strongly recommended to wear gloves during paraffin sectioning and in general to maintain an RNase-free environment during all downstream procedures. Use only heat-treated glassware and RNase-free water. Use SuperFrost®Plus slides drawn directly from new packages.

Workstation and Microtome

Before starting the tissue sectioning, the whole workstation (bench top, microtome, blade holder, brushes, tweezers, cooling plate, water bath etc.) needs to cleaned with RNase-Zap/RNase Away.

Cutting sections

1) Prepare a water bath with room temperature RNase-free water and a warm-water bath with RNase-free water at 40-50°C (depending on the paraffin type).
2) Insert a new disposable blade in the knife carrier and place the paraffin block in the cassette clamp. Trim the block in order to avoid the first couple of sections. It is recommended to cool the FFPE blocks on a cooling plate to app. -15°C before cutting to better control the section thickness.
3) Cut 6 μm-thick paraffin sections and place them in the room temperature RNase-free water, where folding can be reversed. Transfer the sections to the heated water bath, where the tissue section is allowed to stretch shortly. It is recommended to mount sections immediately thereafter on electrostatic treated slides, such as SuperFrost®Plus slides, obtained from a new non-contaminated package.
4) Let the paraffin sections dry for 1-2 hours at room temperature and store at 4°C for up to one week. Avoid melting the paraffin until the day prior to the *in situ* hybridization analysis.
5) Melt paraffin in an oven at 60°C for 45 minutes on the day before conducting the ISH experiment. Store slides overnight at 4°C in an RNase-free environment.

Tip 2

Identify appropriate Proteinase-K treatment range

The degree of Proteinase-K treatment depends on fixation and tissue of origin. In general terms, the harder the fixation, the more Proteinase-K is needed, however there are lower and upper limits. For the Proteinase-K treatment step, it is recommended to vary the concentration or the duration, as indicated in Table 8. Optimal (opt) starting values are shown in parenthesis.

To identify the optimal Proteinase-K conditions, start by testing the LNA™ U6 snRNA probe at 4-5 different concentrations between 0.1-2.0 nM using the One-day microRNA ISH Protocol. Once the conditions have been established, start testing the double-DIG labeled LNA™ microRNA probe (positive microRNA control) and the LNA™ scrambled microRNA probe (negative control).

Table 8

Adjust concentration

	Fixation	Temperature	Proteinase-K, conc (opt)	Duration
human FFPE	routine formalin	37°C	5-20 µg/mL (15)	10 min.
mouse FFPE	PFA perfusion	37°C	0.5 - 5.0µg/mL (2)	10 min.

Adjust duration

	Fixation	Temperature	Proteinase-K, conc	Duration (opt)
human FFPE	routine formalin	37°C	15µg/mL	5-30 min. (10)
mouse FFPE	PFA perfusion	37°C	2µg/mL	3-30 min. (10)

Tip 3

Identify ISH sensitivity level

In order to identify the sensitivity of the performance of the ISH protocol, it is recommended to prepare dilutions of the LNA™ U6 snRNA probe. Figure 3 shows that the LNA™ U6 snRNA probe should provide a significant ISH signal at 0.03 to 0.3nM concentration. It is recommended that U6 snRNA ISH signal is intense (as shown for 0.1 nM concentration in Figure 3), when the probe is incubated at 0.1-2.0 nM before moving on with the double-DIG probes for microRNA ISH.

Figure 3. ***In situ*** **hybridization** (LNA™ U6 snRNA expression) on consecutive sections from FFPE tissue sample from normal human colon.

Once the optimal hybridization conditions are achieved for the LNA™ U6 snRNA probe, it is recommended to use the suggested starting concentrations in Table 9 for the double-DIG labeled miRCURY LNA™ microRNA Detection probes supplied in the microRNA ISH Optimization Kits, (optimal hybridization temperature is in the 50-60°C range).

Table 9. miRCURY LNA™ microRNA Detection probe, suggested concentrations* (positive control as supplied with the Kit)

Probe name	Concentration, nM	RNA T_m
miR-1	20	85°C
miR-21	20-40	83°C
miR-122	40	82°C
miR-124	40	89°C
miR-126	40	85°C
miR-223	40	83°C
miR-145	20	88°C
miR-205	20-40	87°C

*optimization range for the double-DIG LNA™ probe could be 20-80 nM.

Tip 4

Identify optimal hybridization temperature

Optimal performance of an ISH probe is related to its signal-to-noise ratio. Oligonucleotide probes, and especially LNA™ containing probes, can potentially hybridize to highly similar sequences if the hybridization temperature is too low. The positive control LNA™ probes supplied with the microRNA ISH Optimization kit typically result in a high signal-to-noise ratio at 55°C using the One-day miRCURY LNA™ microRNA ISH protocol. The LNA™ probes also hybridize at 60°C, but generally provide weaker signals. At 50°C the LNA™ probes give stronger signals, but the risk of cross-hybridization to highly similar sequences (in RNA transcripts or the genome) will increase at low hybridization temperatures.

As a rule-of-thumb, hybridization should be performed at 30°C below the given RNA T_m (or 20°C below DNA T_m).

Troubleshooting

No signal: If no signal is obtained with the LNA™ U6 snRNA probe incubated at 10 nM, it is recommended to ensure that all reagents are prepared according to the recommendations and are RNase free (pages 14-16). Always test sections from more than one block (a minimum of 4 is recommended).

Not sufficient sensitivity level with the LNA™ U6 snRNA probe. It is recommended that the LNA™ U6 snRNA signal is intense when incubated in the range of 0.1-1 nM. If this is not the case, ensure that the buffers are prepared correctly and that tissue sections are in the range 5-7 µm. Ensure that the AP-reaction takes place at 30°C. Low sensitivity may also be caused by RNase contamination during sectioning or handling during the *in situ* hybridization protocol. Make sure all steps of the ISH protocol are performed in an RNase-free environment. Be aware that both insufficient or hard fixation of tissue samples may result in a low signal. Thus, it is necessary to test several blocks in parallel and avoid concluding on a single sample.

Strong U6 snRNA signal but no or low microRNA signal. If a strong U6 snRNA signal is obtained with 0.1-0.5 nM probe, but no signal is obtained with the supplied positive control LNA™ microRNA probe, it is most likely due to sub-optimal Proteinase-K treatment. Hence, the Proteinase-K concentration or duration of treatment should be optimized (see Tip 2 for details). In order to boost a weak signal, remove the anti-DIG reagent in step 10 halfway through the incubation (e.g. after 30 minutes) and apply new unused reagent for the second half of the incubation. The same approach can be used for the AP substrate in step 12. For low copy number targets it may be possible to increase the signal with Tyramide Signal Amplification (TSA) based systems.

Non-specific staining. It is necessary to clarify whether non-specific staining obtained with the LNA™ scrambled microRNA probe is related to the DIG-labeled probe itself, the detecting antibody or to endogenous enzymatic reactions. This can be done by a systematic approach where the effect of excluding individual reagents is tested including the DIG-labeled probe, the AP-conjugated anti-DIG

or both. If staining is obtained in the absence of AP-conjugated anti-DIG then endogenous AP is present.

If staining is obtained in the absence of the DIG-labeled probe (and no endogenous AP activity is observed) then staining is related to the detecting antibody. If abundant endogenous enzymatic reactivity (e.g. in some intestinal areas and placenta) cannot be prevented by Levamisol, it may require a change to another detection approach, such as TSA-based fluorescence.

Some non-specific staining can be caused by improperly maintained SSC wash buffer temperatures. It is important to ensure that the SSC wash buffers are preheated to and maintained at the hybridization temperature (see step 7, page 20).

High background staining. Providing all possibilities for non-specific staining mentioned above have been ruled out and as long as the specific signal from the microRNA probe is strong, high background signal can often be reduced by increasing the hybridization temperature and/or increasing the duration of the stringency washes.

Non-specific staining of ECM. Non-specific staining of extracellular matrix may occur if the concentration of the detecting antibody is too high.

Sections fall off after de-paraffination. Avoid storage of paraffin sections at -20°C. Small and thick sections fall off more easliy than large thin sections. Ensure that the glass slides used have electrostatic properties such as the SuperFrost®Plus slides. When transferring sections from the waterbath to slides it is important to let all excess water drain/evaporate from the section and slide to avoid water or air bubbles getting trapped under the section. For fatty tissues or loose connective tissue e.g. tissue from normal breast, increasing the duration of the melting step from 45 minutes to 60 minutes sometimes helps.

Frequently asked questions

Can I use a hybridization oven instead of a Dako Hybridizer? When using a hybridization oven during the hybridization step, make sure cover-slides are sealed with Fixogum as specified in step 6. The slides can be placed as such in the hybridization oven without humidifying conditions. However, humidifying conditions may be tried, e.g. by using 1xSSC. In order to establish a more stable hybridization temperature place a metallic plate, e.g. the inserts from a multiblock heater, in the oven. Place the slides on the plate and hybridize for 1–2 hours. Then go to step 6.

Can I use the protocol for Fresh frozen tissue? The protocol can be adapted to cryosections, please find guidelines for fresh frozen samples at www.exiqon.com/mirna-ish-kit. The protocol will require optimization for individual sample types and microRNA targets.

Can I pause the ISH procedure? The individual steps in the One-day protocol have been optimized to accommodate a One-day protocol. PBS steps may be prolonged, but it is not recommended to extend to protocol to more than one day.

What happens if sections dry out? Sections should be maintained in buffered solutions after the hybridization step. Tissue sections that dry out may cause protein denaturation, which may be particularly harmful to the detecting antibody and its conjugated alkaline phosphatase. This may lower the sensitivity of the assay significantly and in addition cause background staining. Drying out of tissue sections may also reduce the quality of the tissue morphology.

Can I use other detection methods? The DIG labeled LNA™ probes can be detected using alternative methods for DIG detection such as the TSA based systems. The use of alternative systems may necessitate additional steps to be added at various stages of the protocol and requires optimization. Please go to www.exiqon.com/mirna-ish-kit for more information.

References

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- www.exiqon.com

Outside North America
Exiqon A/S · Skelstedet 16
DK-2950 Vedbaek · Denmark
Phone +45 45 660 888
Fax +45 45 661 888

North America
Exiqon Inc. · 12 Gill Street, Suite 1650
Woburn, MA 01801 · United States
Phone (781) 376 4150
Fax (781) 376 4152

exiqon.com

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